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UNITED STATES PATENT APPLICATION

OF

CHRISTOPH WAGENER

FOR

INFLUENCING ANGIOGENESIS USING CD66a

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is filed under the provisions of 35 USC §371 and §111 and claims the priority of International Patent Application No. PCT/DE99/03671 filed November 16, 1999, which in turn claims priority of German Patent Application No. 198 52 804.3 filed November 16, 1998.

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INFLUENCING ANGIOGENESIS USING CD66a

- [001] The invention relates to a pharmaceutical composition for influencing angiogenesis. In one case, angiogenesis may be improved by administration of CD66a or substances initiating the formation of CD66a, while in the other case angiogenesis may be inhibited by using substances preventing interaction between CD66a and CD66a ligands.
- [002] The formation of blood vessels (angiogenesis) is in many diseases an important step which may contribute to curing, on the one hand, or shall desirably be prevented in other cases. Improving angiogenesis is very desirable e.g. for cardiovascular diseases to treat angina pectoris or heart attacks or cerebral infarctions, for example. On the other hand, the inhibition of the vascular supply of malignant solid tumors in humans and animals is a promising approach in tumor therapy. Angiogenesis inhibitors, such as endostatin, directly attack normal and thus genetically stable endothelial cells of the blood vessels supplying a tumor, cause them to die off and thus stop the supply of the tumor cells with nutrient-containing blood (cf. Kerbel, R., Nature, 390, p. 335 et seq., 1997). This leads to a regression of blood vessels and tumor mass. Since contrary to the tumor cells the endothelial cells are genetically stable, resistances do not form as is the case e.g. in a cytostatic therapy aiming directly at the tumor cells. By inhibiting angiogenesis the growth of human tumors could be blocked in experimental models. Some angiogenesis inhibitors are meanwhile tested clinically (cf. Hanahan *et al.*, Cell 86, 353-364, (1996)).
- [003] The supply of tissues with new vessels is a complex process in which a number of biomolecules are involved. Tumors produce soluble mediators, for example, which initiate the formation of new vessels. When angiogenesis proceeds, adhesion molecules play a central part. They control the communication of vessel cells with one another and with the surrounding connective tissue. Finally, various proteinases are also involved in the neovascularization.
- [004] It is the object of the present invention to provide a possibility of improving or inhibiting angiogenesis as desired. In case angiogenesis is inhibited, a form of cancer therapy without the development of resistances shall thus be provided, i.e. in particular tumor-accompanying angiogenesis shall be influenced within the meaning of a reduction of inhibition.
- [005] According to the invention this is achieved by the subject matters defined in the claims.

[006] The subject matter of the present application is in particular a pharmaceutical composition suitable to regulate angiogenesis. Such a composition comprises:

- (a) for positive regulation  
one or more bodies of  
CD66a, CD66a fragments or CD66a-derived glycostructures, or CD66a ligands, ligand fragments or structures derived therefrom, as well as substances inducing the expression of CD66a or CD66a ligand,

or

- (b) or negative regulation  
one or more bodies of  
substances which inhibit the interaction between CD66a and CD66a ligands or substances which inhibit the expression of CD66a or CD66a ligand.

[007] The protein CD66a which is also referred to as biliary glycoprotein (BGP), transmembrane carbonembryonic antigen or human C-CAM is a special adhesion molecule. The term CD66a is used below. The gene coding for CD66a has already been cloned (Hinoda *et al.*, PNAS 85, 6959-6963, 1988). The applicants of the present application described in 1991 already the only CD66a-specific monoclonal antibody world-wide (Drzeniek *et al.*, Cancer Letters 56, 173-179 (1991); Stoffel *et al.*, J. Immunol. 150, 4978-4984 (1993)). This antibody is referred to as 4D1/C2 and was deposited with DSMZ (*Deutsche Sammlung von Mikroorganismen und Zellkulturen* [German-type Collection of Microorganisms and Cell Cultures], Mascheroder Weg, Braunschweig, under accession number DSM ACC2371 on October 22, 1998.

[008] It has now been found surprisingly that the CD66a factor is expressed in tumor capillaries whereas the blood vessels of the corresponding normal tissues are negative.

[009] In a human Leydig cell tumor, the individual stages of the neovascularization could be traced accurately. In this connection, it was found that certain stages of neovascularization can be correlated with the occurrence of the following factors:

1. proliferation of endothelial cells: VEGF (vascular endothelial growth factor), VEGF receptors
2. formation of vascular lumens: CD66a

3. next differentiation step: endostatin
4. next differentiation step: angiostatin

[0010] In recent experiments conducted by the inventors using chicken embryos it could be shown that CD66a is a potent angiogenetic factor and improves the neovascularization of normal and tumoral tissues.

[0011] It could also be shown that CD66a was blocked by an antibody directed against CD66a and the formation of capillaries necessary for tumor growth is inhibited. Tumor growth can no longer take place.

[0012] CD66a can be detected in human tumors in newly formed blood vessels (capillaries) in a defined differentiation window, namely in the stage of lumen formation. In an *in vitro* differentiation model a monoclonal CD66a antibody inhibits the formation of vascular tubes (tube formation) by human endothelial cells. These results prove that CD66a plays an essential part in angiogenesis. It follows from the expression of CD66a in tumoral vessels and the *in vitro* inhibition of capillary structure formation by a monoclonal CD66a antibody that tumor angiogenesis can be inhibited by functionally blocking CD66a.

[0013] Experiments conducted with transfectomas have shown that CD66a binds to itself (homeotypical binding) and to other members of the CD66 family. The localization of CD66a in newly formed endotheliums at the basal cell pole and the inhibition of capillary formation indicate that CD66a interacts with components of the extracellular matrix.

[0014] Antibodies, peptides, proteins or other agents which bind specifically to one or more functional domains of CD66a or its ligands are particularly suitable for the CD66a inhibition desired according to the invention in one respect. Monoclonal antibodies which are directed against adhesive, functionally significant domains of CD66a are preferably used. Furthermore, CD66a has glycostructures which may have an angiogenetic effect, e.g. LewisX and sialyl-LewisX groups. The above-mentioned monoclonal CD66a antibody 4D1/C2 is preferably used. This leads to an inhibition of tumor angiogenesis via a functional inactivation of CD66a. CD66a is in this connection functionally inactivated by inhibiting the interaction between CD66a and possible ligands. Here, structures which mediate interaction are blocked. Furthermore, soluble ligands or soluble ligand domains may also be used to block the interaction. The invention also relates to the use of recombinant domains which correspond to a CD66a fragment and to fragments of antibodies which react substantially with the epitope of CD66a. As a result, the signal chain starting from CD66a is blocked. The employed compounds may also be modified suitably to bind e.g. irreversibly to the receptor.

[0015] In particular CD66a as a whole molecule, CD66a domains as well as specific glycostructures of CD66a are suitable for the angiogenesis improvement desired according to the invention in one respect. In these cases, the soluble molecule form is applied to the sites of the body where angiogenesis shall be triggered (e.g. in the cardiac muscle). A DNA may also be used which codes for CD66a or parts of the CD66a protein. The DNA may also be integrated in vectors which are common in gene therapy (e.g. adenoviruses). Synthesis of the protein may also be achieved by administration of simple plasmid DNA. The positive influence of angiogenesis is effected by improving interactions between CD66a and CD96a ligands.

[0016] Methods of obtaining the above-mentioned antibodies which may be used for inhibiting angiogenesis are known to a person skilled in the art and comprise e.g. as to polyclonal antibodies the use of CD66a or a fragment thereof as immunogen for immunizing suitable animals and obtaining serum. The person skilled in the art is also familiar with methods of producing monoclonal antibodies. For this purpose, e.g. cell hybrids are produced from antibody-producing cells and bone marrow tumor cells (myeloma cells) and cloned. Thereafter, a clone is selected which produces an antibody specific to CD66a. This antibody is then produced according to standard methods. Examples of cells which produce antibodies are spleen cells, lymph node cells, B lymphocytes, etc. Examples of animals which may be immunized for this purpose are mice, rats, horses, goats and rabbits. The myeloma cells may be obtained from mice, rats, humans or other sources. The cell fusion may be carried out e.g. by the generally known method of Köhler and Milstein. The hybridomas obtained by cell fusion are screened using CD66a according to the enzyme-antibody method or according to a similar method. Clones are obtained e.g. with the boundary dilution method. The resulting clones are implanted intraperitoneally into BALB/c mice. Ascites is removed from the mouse after 10 to 14 days, and the monoclonal antibody is purified by known methods (e.g. ammonium sulfate fractionation, PEG fractionation, ion exchange chromatography, gel chromatography or affinity chromatography). The collected antibody may be used directly or a fragment thereof may be employed. In this connection, the term "fragment" means all parts of the antibody (e.g. Fab, Fv or single chain Fv fragments) which have an epitope specificity the same as that of the complete antibody.

[0017] In one embodiment, said monoclonal antibody is an antibody originating from an animal (e.g. mouse), a humanized antibody, a chimeric antibody or a fragment thereof. Chimeric antibodies which are similar to human antibodies or humanized antibodies have a reduced potential antigenicity but their affinity over the target is not lowered. The production of chimeric and humanized antibodies or of antibodies similar

to human antibodies was discussed in detail (Noguchi, Nippon Rinsho, 1997, 55(6) pp. 1543-1556; van Hogezaand, Scand. J. Gastroenterol. Suppl., 1997, 223, pp. 105-107). Humanized immunoglobulins have variable framework regions which originate substantially from a human immunoglobulin (designated acceptor immunoglobulin) and the complementarity of the determining regions which originate substantially from non-human immunoglobulin (e.g. from mouse) (designated donor immunoglobulin). The constant region(s) originate(s), if available, also substantially from a human immunoglobulin. When administered to human patients, humanized (and human) anti-CD66a antibodies according to the invention offer a number of advantages over antibodies from mice or other species: (a) the human immune system should not regard the framework or the constant region of the humanized antibody as foreign and therefore the antibody response to such an injected antibody should be less than that to a fully foreign mouse antibody or a partially foreign chimeric antibody; (b) since the effector region of the humanized antibody is human it might interact in a better way with other parts of the human immune system, and (c) injected humanized antibodies have a half life substantially equivalent to that of naturally occurring human antibodies, which permits administering smaller and less frequent doses as compared to antibodies of other species.

[0018] The above described conventional technology may also be supplemented or replaced using recombinant phage libraries (Felici *et al.*, Biotechnol. Rev. 1, pp. 149-183 (1995); Hoogenboom *et al.*, Immunotechnology 4, pp. 1-20 (1998)). Recombinant phage libraries may have random peptide structures in the antigen-binding regions of the phage-presented antibody fragments. The advantage of this technology is *inter alia* that in cloned phages the information on the amino acid sequence of the antigen binding structures is directly available.

[0019] The domains of CD66a or the CD66a ligands, whose blocking effects a functional inactivation of CD66a, may be recombined in any way and be used while being introduced into molecules which are suitable for therapeutic purposes (e.g. to achieve better immunological compatibility). The reactive domains may also be expressed according to molecular-biological standard methods, e.g. bacterially or in insect cells.

[0020] Interaction between CD66a and potential ligands may preferably be inhibited in the following ways (negative regulation):

- inhibition by antibodies and antibody fragments against the functional domain of CD66a,
- inhibition by antibodies and antibody fragments against the functional domains of the CD66a ligands,
- inhibition by the functional domain of CD66a,

- inhibition by the functional domain of the CD66a ligands,
- inhibition of the endogenous formation of CD66a or CD66a ligands using anti-sense oligonucleotides.

[0021] Interaction between CD66a and potential ligands may preferably be improved in the following ways (positive regulation):

- application of the native molecule purified by means of biochemical methods,
- application of recombinant CD66a fragments,
- application of angiogenetically active glycostructures isolated from CD66a,
- application of glycostructures prepared in a fully synthetic or partially synthetic way, whose structure was derived from angiogenetically active glycostructures of CD66a,
- application of a DNA, which codes for the complete CD66a protein thereof, in the form of suitable vectors or plasmids,
- application of a DNA, which codes for isoforms or fragments of CD66a, in the form of suitable vectors or plasmids.

[0022] The pharmaceutical compositions according to the invention may be administered in any way suitable to reach the desired tissue. The administration is preferably carried out parenterally, particularly orally, intravenously or intratumorally. For the purpose of administration, the substance is used in a formulation suitable for the respective kind of administration using corresponding common pharmaceutical excipients. Orally applicable pharmacons are developed in two ways. On the one hand, interaction between ligand and receptor may be modelled e.g. by X-ray structural analysis or NMR spectroscopy. On the other hand, chemical combinatorial libraries (Myers, Current Opinion in Biotechnology 8, pp. 701-717 (1997) may be used. Here, the interaction of the ligand or receptor is examined with initially largely randomly combined chemical compounds. If binding was detected, the binding properties can be defined in more detail by selecting similar compounds.

[0023] Dosage and posology of the administration of the compounds according to the invention are determined by a physician on the basis of the patient-specific parameters, such as age, weight, sex, severity of the disease, etc.

[0024] According to the kind of administration, the medicament is formulated suitably, e.g. in the form of solutions, suspensions, as a powder, tablet or capsule or injection preparations which are produced according to common galenic methods.

[0025] The infusion or injection solutions are preferably aqueous solutions or suspensions, it being possible to produce them prior to use, e.g. from lyophilized preparations which contain the active substance as such or together with a carrier, such as mannitol, lactose, glucose, albumin or the like. The ready-to-use solutions are sterilized and optionally mixed with auxiliary agents, e.g. with preservatives, stabilizers, emulsifiers, solubilizers, buffers and/or salts for regulating the osmotic pressure. The sterilization may be obtained by sterile filtration through filters having a small pore size, whereupon the composition may optionally be lyophilized. Antibiotics may also be added to help maintaining sterility.

[0026] The pharmaceutical compositions contain a therapeutically active amount of one or more of the above-mentioned active substances together with common auxiliary agents and carrier substances. They are preferably organic or inorganic liquid pharmaceutically compatible carriers which are suitable for the desired administration and which do not interact negatively with the active components.

[0027] The pharmaceutical preparations according to the invention are sold as unit dosage forms, e.g. as ampoules.

[0028] The invention also relates to a method of producing a pharmaceutical composition, which is characterized in that the compound according to the invention is mixed with a pharmaceutically compatible carrier.

[0029] "Substances inhibiting the expression of CD66a or CD66a ligand" are administered preferably by means of gene therapy introducing into tumor cells e.g. anti-sense oligonucleotides to CD66a and/or CD66a ligand. These oligonucleotides are derived from the known sequences for CD66a or CD66a ligand (Hinoda *et al.*, Proc. Natl. Acad. Sci. U.S.A. 85, p. 6959 (1988)). The anti-sense oligonucleotides may also reach the size of a DNA which is complementary to regions of the gene mRNA and binds thereto. Then, a duplex molecule forms which is taken away from the translation of the mRNA. Inhibition of the gene expression can be achieved in this way. The term "anti-sense oligonucleotide" comprises any DNA or RNA molecule which is complementary to regions of the CD66a RNA or CD66a ligand RNA, in particular mRNA and most particularly regulatory elements thereof, and effects inhibition of the gene expression by binding to these regions. The anti-sense oligonucleotides may be available as such or, if they are relatively long, in the form of a vector or vector construct coding for them, which is sometimes also referred to as "minigene". Such a vector may be a common expression vector. It may be favorable for the expression of the sequence coding for them to be controlled by a constitutive or inducible



promoter, such as a tissue-specific or tumor-specific promoter. The anti-sense molecules may be introduced by common methods. If the anti-sense oligonucleotides are available as such or in the form of a vector coding for them, e.g. transfection techniques or packaging in liposomes is suitable.

[0030] "Substances which induce the expression of CD66a or CD66a ligand" are e.g. DNA molecules which code for CD66a or angiogenetically active CD66a fragments or for CD66a ligands or angiogenetically active ligand fragments. The expression is controlled by suitable regulatory sequences. The DNA is administered according to protocols known to a person skilled in the field of gene therapy. Thus, e.g. packaging of the DNA in viral particles (e.g. adenoviruses) or the administration of naked plasmid DNA is in consideration.

[0031] According to the invention the growth of all solid tumors of the body may be inhibited with the angiogenesis-inhibiting pharmaceutical composition. Examples are epithelial tumors (e.g. squamous epithelium, columnar epithelium, glandular epithelium, transitional epithelium), mesenchymal tumors (e.g. fibers, muscles, cartilages, and bone tissues), mixed tumors (mixed epithelial, mixed mesenchymal, epithelial-mesenchymal), tumors of the hematopoietic and lymphatic tissues (bone marrow, lymphatic tissue), tumors of the serous cavities (e.g. pulmonary pleura, heart sac, abdominal membrane, synovial membrane), tumors of the nervous system (e.g. ganglion cells, neuroepithelium, neuroglia, meninges, sympatheticus, peripheral nerves), tumors of the gastro-intestinal tract and tumors of individual organs. The growth of tumors of the bronchi and the lungs, breast, liver, bile, pancreas, kidneys and urinary tracts, stomach, large intestine, straight intestine, prostate and uterus are preferred according to the invention.

[0032] According to the invention the neovascularization may be induced by the angiogenesis-improving pharmaceutical composition in diseases in which the disease-dependent occlusion of vessels results in a insufficient supply of the tissue with oxygen and nutrients. Cardiac diseases or insufficient blood supplies of the extremities in diabetics, heavy smokers or patients suffering from hypertension are to be mentioned as examples.

[0033] The invention is described in more detail by means of the figures:

Figure 1            Localization of CD66a in the vessels of a human Leydig cell tumor. The immunohistochemical staining was carried out using the 4D1/C2 antibody.

- Figure 1a: One of the stained tumor capillaries is marked by an arrow (x350)
- Figure 1b: Enlargement of a region from figure 1a. The arrow points to the staining of an endothelial cell (x950).
- Figure 2 Chemotactic effect of CD66a (= BGP) on HDMEC
- Figure 3 Proliferation of HDMEC following stimulation using CD66a (= BGP)
- Figure 4 Effect of CD66a on the formation of capillary-like vascular tubes in cell culture

[0034] The invention is explained in more detail by means of the following examples.

#### EXAMPLE 1

##### Localization of CD66a in tumor capillaries

[0035] Tumors were stained immunohistochemically using the monoclonal anti-CD66a antibody 4D1/C2 and investigated by means of an optical microscope. For this purpose, an intensifying method using nickel and glucose oxidase was used in addition to the previously employed immunohistochemical methods (Prall *et al.* (1996), J. Histochem. Cytochem. 44, 35-41). Furthermore, electron-microscopic analyses were carried out following immunohistochemical staining using the monoclonal 4D1/C2 antibody (see figure 1).

[0036] Human testicular tumors, brain tumors as well as prostate, bladder and kidney carcinomas were examined immunohistochemically. CD66a was localized in endothelial cells and in the basal membrane of the tumor capillaries. Mature, non-proliferating resting vessels of the examined organs were negative. In case the tumor is divided into different zones in accordance with functional aspects, namely tumor cells, tumor margin and tumor environment, the positive immune response can be found in the newly formed tumor capillaries on the tumor margin. This indicates a function of CD66a in very early stages of neovascularization (neangiogenesis).

#### EXAMPLE 2

##### Effect of CD66a on the proliferation and chemotaxis of cultured endothelial cells

- [0037] In order to test the effect of CD66a on the proliferation and chemotaxis of cultured endothelial cells, the glycoprotein was purified from membrane fractions of human granulocytes. The membrane fraction was isolated in accordance with established methods (Drzeniek *et al.* (1991), Cancer Letters 56, 173-179; Stoffel *et al.* (1993), J. Immunol. 150, 4978-4984). After extracting the membrane glycoproteins with a non-ionic detergent, they were bound to an immobilized monoclonal CD66 antibody and eluted using glycine-HCl at pH 2.2. Following neutralization the eluate was further separated by means of gel chromatography on Superdex 200 (Pharmacia). The CD66a-positive fractions were pooled. Contaminations in the low-molecular region were separated by means of ultrafiltration using a filter having an exclusion of 100 kD. In combination with a Western blot it was shown by means of SDS-PAGE in silver gel that the supernatant exclusively contained CD66a. This fraction was used for cell culture experiments with endothelial cells.
- [0038] The experiments were carried out with two different human endothelial cell forms, namely with HUVEC (human umbelical vein endothelial cells) and HDMEC (human dermal microvascular endothelial cells).
- [0039] The effect of CD66a on the proliferation was checked in a monolayer culture. Endothelial cells were seeded in a defined number on a microtitration plate. After 72 hours, the number of endothelial cells in stimulated and non-stimulated cultures was compared. It turned out that CD66a stimulated the proliferation of both cell lines in dose-dependent manner.
- [0040] The effect of CD66a on chemotaxis was investigated in a two-chamber culture system (what is called a Boyden chamber). The cells are cultured in the top chamber. The bottom chamber contains chemotactic substances. Both chambers are separated by a polycarbonate filter permitting passage of the cells. After adding CD66a to the bottom chamber, a dose-dependent chemotactic effect showed on both endothelial cell lines. The effect of CD66a could be compared with the effect of VEGF. As evident from figure 2, CD66a (= BGP) has a chemotactic effect from a concentration of 100 ng/ml. With a concentration of 150 ng/ml the chemotactic effect is only slightly less than that of VEGF (vascular endothelial growth factor).
- [0041] The chemotactic effect of CD66a was also analyzed in combination with VEGF and bFGF (basic fibroblast growth factor). The chemotactic effect of VEGF or bFGF was increased by CD66a by about 30 % each.

[0042] Cultured human microvascular dermatofibroblasts were incubated with CD66a (= BGP) in concentrations of 50, 100, 200, 400 and 600 ng/ml. From a concentration of 200 ng/ml a proliferation-increasing effect of CD66a could be detected. This is shown in figure 3.

[0043] Due to the positive effect on proliferation and chemotaxis CD66a fulfills the main criteria of angiogenesis factors.

### EXAMPLE 3

[0044] Effect of CD66a on the formation of capillary-like vascular tubes in cell culture.

[0045] The test results described in Example 2 suggest that CD66a is causally involved in the formation of new vessels (neoangiogenesis). In order to check this hypothesis, animal experiments would be most suitable. However, since CD66a is a human glycoprotein, it has to be expected that due to the differences in the species the effect in the experimental animal shows no or only slight expression. The finding that the monoclonal anti-CD66a 4D1/C2 antibody shows good reaction in human tissues supports this assumption. The reaction is weak in the corresponding tissues of rats and mice and can be distinguished only with difficulty from a non-specific background reaction. The 4D1/C2 antibody obviously binds to an antigenic structure which does not occur in rodents in this form.

[0046] In order to circumvent the problems caused by the differences regarding the species, cell culture models are used in which endothelial cells grow under certain conditions into vascular tubes which correspond to newly formed capillaries (tube formation). For this purpose, the cells are cultured in the presence of specific growth factors such as VEGF (vascular endothelial growth factor) or FGF-2 (fibroblast growth factor) in a connective tissue matrix. This culture form represents a good approach to *in vivo* conditions.

[0047] In order to investigate the significance of CD66a for the formation of capillaries, HUVEC and HDMEC cells were cultured in three-dimensional collagen I gels. In the presence of growth factors such as VEGF and FGF-2, the endothelial cells form tubular structures which correspond to newly formed capillaries. In the presence of the monoclonal CD66a 4D1/C2 antibody, the formation of vascular tubes was inhibited. A second monoclonal antibody which is directed against another epitope on CD66a, had no effect on the formation of tubes. These experiments prove a functional correlation between the expression of CD66a and the neoformation of capillary-like vascular tubes. The functional domain of CD66a is also defined by means of the antibody.

[0048] The results of the above experiments are shown in figure 4:

[0049] In the presence of the angiogenesis factor VEGF (50 ng/ml), capillary-like structures develop (see figure 4a). Capillary-like structures manifest themselves by way of tubes in which the longitudinal endothelial cells are arranged parallel. These tubes can be compared to fish schools. In the middle of figure 4a there is a region in which the endothelial cells are rounded. They are no tubes.

[0050] Figure 4b shows the result of an experiment in which the capillary formation was investigated in the presence of VEGF (50 ng/ml) and CD66a (150 ng/ml). As compared to figure 4a, almost all endothelial cells are involved in the formation of tubes. Furthermore, a branching pattern can be seen which supports the further differentiation of the angiogenesis process. CD66a thus intensifies the angiogenetic effect of VEGF.

[0051] In figure 4c, the endothelial cells were cultured in the presence of CD66a (300 ng/ml) and in the absence of VEGF. Capillary-like structures appear.

[0052] Figure 4d shows the result of an experiment in which the endothelial cells were cultured in the presence of the monoclonal 4D1/C2 antibody. The formation of capillaries is fully inhibited. It follows from this experiment that the 4D1/C2 antibody binds to a domain of CD66a which is essential for the formation of capillaries.